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Genotype F Prevails in HBV Infected Patients of Hispanic Origin in Central America and May Carry the Precore Stop Mutant

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The distribution of HBV genotypes and the presence of the precore stop mutation were investigated in HBV strains from Central America. 333 HBsAg positive sera from chronic HBsAg carriers and acute hepatitis B cases from five different countries (Costa Rica, Nicaragua, Honduras, El Salvador and Guatemala) were tested for HBV DNA by nested PCR. Genotyping by limited sequencing within the S gene was performed on 90 strains, 66 from sera with a high level of HBV DNA, and another 24 from sera positive for HBV DNA only after nested PCR. 23 of the samples were anti-HBe positive. Genotype F was found in 71 (79%), A in 13 (14%), D in 5 (6%) and C in one of the 90 sera. 18 patients with genotype F infection had anti-HBe and HBV DNA in serum. Since the three published precore sequences of genotype F strains have a C^{1858}, which is known to prevent the precore stop mutation from G to A at position 1896, the precore and part of the core genes were sequenced from 19 anti-HBe positive sera with HBV DNA, 17 with genotype F and 2 with genotype A. The A^{1896} mutation was found in 11 of the 17 genotype F strains. All these had a T^{1858}, which was also present in 5 of the 6 genotype F strains with G^{1896}. The precore region was therefore sequenced from genotype F strains from 5 HBeAg positive sera from the five different Central American countries. These also had a T^{1858}, which thus is the wild type substitution in genotype F in Central America. A number of mutations were recorded between residues 57 and 68 in the core protein corresponding to a unique clustering region of the genotype F strains. The predominance of genotype F in Central American populations of Hispanic origin was not anticipated since this genotype is regarded as indigenous to the Amerindian populations of the New World. ***J. Med. Virol. 51:305–312, 1997.***

KEY WORDS: HBV genotype; Central America; core mutations

INTRODUCTION

The study of genotypes is now an important area of research for many different viruses, for example HCV (Simmonds et al., 1994), and HIV (Salminen et al., 1993), but so far less attention has been paid to the genotypes of HBV. The virus may be classified into six genotypes, A to F (Okamoto et al., 1989; Norder et al., 1992). The global molecular epidemiology of HBV has been revealed by comparing the S genes from a large number of HBV strains of diverse geographical origin (Norder et al., 1993). The genotypes have a characteristic geographical distribution and are in general linked to the HBsAg subtype. Thus, strains belonging to genotype A are mainly found in Northern Europe, USA and sub-Saharan Africa. Genotypes B and C are found in South-East Asia and the Far East. Genotype D is found worldwide, but is the dominating genotype of the Mediterranean area, the Near and Middle East and South Asia. Strains belonging to genotype E have so far only been reported from sub-Saharan Africa. There are indications that genotype F encoding *adw4* represents the HBV strains of the Amerindian populations of the Americas (Norder et al., 1993). Data regarding HBV genotypes in Latin America are still scanty (Niel et al., 1994), although some conclusions may be inferred from the HBsAg subtype distribution (Castillo et al., 1979; Gaspar et al., 1987). So far there have been no reports on HBV genotypes in Central America.

HBeAg is an established marker of viral replication and infectivity, and seroconversion to anti-HBe is in general associated with clearance of serum HBV DNA

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and remission of liver disease (Hoofnagle et al., 1981). However, some patients remain HBV DNA positive despite seroconversion to anti-HBe, and these HBeAg negative replicating variants may be associated with both severe liver disease (Brunetto et al., 1991; Omata et al., 1991) and fulminant hepatitis (Liang et al., 1991; Kosaka et al., 1991; Carman et al., 1993; Hasegawa et al., 1994). Sequencing of viral DNA from such cases has revealed the presence of several different mutations in the precore and core regions of these strains. One of the most common mutations is a G to A transition at position 1896, which produces a change from Trp^{28} (TGG) to a translational stop codon (TAG) in the precore abrogating the HBeAg synthesis (Brunetto et al., 1989; Carman et al., 1989; Okamoto et al., 1990). This stop mutation functions as an immune escape mutant, since it prevents the expression of HBeAg on the surface of HBV infected hepatocytes, and therefore protects these from being eliminated by cytotoxic T cells specific for HBeAg/core, which cross-react at the T cell level (Milich et al., 1986). The emergence of HBV precore mutants in anti-HBe positive chronic hepatitis B appears to vary between different areas of the world (Li et al., 1993). The presence of other mutations/substitutions in the pregenomic encapsidation signal in the precore region has been documented in HBV variants unable to express HBe protein, mainly in those with a stop at codon 28 (Tong et al., 1990; Carman et al., 1992; Laskus et al., 1994; Lok et al., 1994). Recently, the different genotypes of HBV have been shown to differ with regard to their association with precore stop mutants, depending on the substitution of position 1858 which opposes position 1896 in the stem of the pregenomic encapsidation signal (Li et al., 1993). Thus in genotype A the presence of a C^{1858} prevents the G to A mutation at 1896, since it will destabilize the stem of the encapsidation signal. Apart from in genotype A a C^{1858} has been found only in three sequenced precore regions of genotype F strains (Naumann et al., 1993; Norder et al., 1994). Therefore, it is of interest to investigate the presence of precore mutants in genotype F strains. The aims of this study were, therefore, to determine which HBV genotypes occur in Central America, and also to determine the presence of HBV variants in the precore/core region in different genotypes.

MATERIALS AND METHODS

Serum Samples

333 HBsAg positive sera from five different countries in Central America (Guatemala, El Salvador, Honduras, Nicaragua and Costa Rica) kept at the International Center of Medical Research and Training in Costa Rica (LSU-ICMRT) were investigated. These belonged to 4 different groups of patients. 1) 105 sera from blood donors found HBsAg positive at screening and referred to ICMRT for confirmation, 2) 156 sera from patients with clinical and laboratory diagnosis of hepatitis B. 71 sera derived from acute cases based on clinical criteria and a positive anti-HBc IgM test, and 85 sera derived from chronic cases diagnosed by follow-up and/or serological criteria, 3) 63 sera from pregnant females (mean age 26 years, range 17 to 41) found HBsAg positive at their first prenatal visit, 4) 9 sera from Amerindians, 8 of which were found HBsAg positive in a study on the prevalence of serological markers for different infectious agents including HIV, HCV, HTLV I-II, *Trypanosoma cruzi* and HBV in 693 Amerindians. In study groups 1 to 3 HBeAg positive sera were preferentially selected to simplify genotyping by sequencing. All sera had been collected by local health systems through Central America (International Red Cross and local hospitals) between 1972 to 1994 and had been stored at −20°C in the LSU-ICMRT.

Serology

Serological tests for HBsAg, HBeAg, anti-HBe, total anti-HBc and anti-HBc IgM, were carried out by in-house radioimmunoassays (RIAs) (LSU-ICMRT). The RIAs were all performed in Immunolon 2 Removawell microtitre plates (Dynatech Laboratories, Inc. Chantilly, Va.), whereafter single wells were transferred to tubes and measured in a gamma counter. 10 mM sodium bicarbonate buffer pH 9.6 and PBS with 0.5% Tween 20 were used as coating buffer and washing buffer, respectively. For capturing antigen to antibody coated solid phase buffer D (0.15 M PBS with 5% fetal calf serum, 1% 0.1 M EDTA and 0.5% Tween 20) was used. IgG was purified by affinity chromatography on protein A columns. Radiolabelling was performed with ^{125}I using the chloramine T method (Hunter et al., 1978). 100,000 cpm were used in all assays. 2.2 times the negative controls and 50% blocking were used, respectively, as cut-off in the HBsAg RIA and in the blocking assays. The cut-off in the assays for anti-HBc IgM and HBeAg was based on the mean of the negative controls plus 10% of the mean for the positive control. 100 μl of serum dilution to be tested or of reagent were used throughout. Incubations were overnight at room temperature or for one to three hours at 40°C. The specific reagents used in the different assays are described below.

HBsAg

Goat anti-HBs IgG (Accurate, Westbury, NY, USA) diluted 1:2,000 was used for coating. Sera were tested undiluted. Bound antigen was indicated with ^{125}I-rabbit IgG, purified from rabbit sera with a rheophoresis (RP) titer $\geqslant 32$. The rabbits were immunized with plasma derived HBV vaccine (HB-Vax, MSD).

HBeAg/anti-HBe

Human IgG anti-HBe (RP titer 1:32) diluted 1:200 was used for coating. In the HBeAg test the sera were diluted 1:2 in buffer D. ^{125}I human anti-HBe IgG (RP titre 1:32 before purification) diluted in buffer D was used to indicate bound antigen. The anti-HBe IgG used in this step was different to the one used for coating.

TABLE I. Detection of HBV-DNA by Nested PCR in 333 HBsAg Positive Sera from Central America

	≥10 pg HBV DNA/ml	≥ 0.1 pg HBV DNA/ml
Blood donors (n = 105)		
HBeAg/Anti-HBe		
(+/–)	21/37 (57%)	33/37 (89%)
(–/–)	0/12	2/12 (17%)
(–/+)	1/56 (2%)	26/56 (46%)
Patients (n = 156)		
Acute (n = 71)		
HBeAg/Anti-HBe		
(+/–)	12/40 (30%)	37/40 (92%)
(–/–)	0/9	4/9 (44%)
(–/+)	1/22 (5%)	7/22 (32%)
Chronic (n = 85)		
HBeAg/Anti-HBe		
(+/–)	22/39 (56%)	38/39 (97%)
(–/–)	0/3	0/3
(–/+)	0/43	9/43 (21%)
Pregnant (n = 63)		
HBeAg/Anti-HBe		
(+/–)	9/12 (75%)	12/12 (100%)
(–/–)	0/9	3/9 (33%)
(–/+)	0/42	6/42 (14%)
Red Indians (n = 9)		
HBeAG/Anti-HBe		
(+/–)	—	—
(–/–)	0/1	0/1
(–/+)	0/8	0/8
Total	66/333 (20%)	177/333 (53%)

For the anti-HBe test 50 μl of an HBeAg positive serum (RP titer 1:32) diluted 1:35 in buffer D was mixed with the same volume of serum to be tested. ^{125}I-anti-HBe IgG (RP titer 1:32 before purification) diluted in buffer D was thereafter added to indicate residual HBeAg on the solid phase.

Total anti-HBc

Human anti-HBc IgG diluted 1:3,000 was used for coating. The IgG was produced from a pool of 5–10 HBsAg and anti-HBc positive sera with an immune adherence (IA) titer ⩾51,200. Core antigen purified from Dane particles diluted 1:40 in buffer D was captured onto the solid phase. Ten μl of serum diluted 1:100 in buffer D were mixed with 90 μl ^{125}I-anti-HBc IgG, purified from a pool of 5–10 human sera different from those used for core antigen capture.

Anti-HBc IgM

Anti-human IgM (Dako) diluted 1:1,000 was used for coating. Sera to be tested were diluted 1:100 in dried milk solution. Core antigen diluted in buffer D was captured onto solid phase, and bound antigen was indicated with ^{125}I-human anti-HBc IgG in buffer D.

Amplification by PCR and Sequencing

DNA extraction and PCR were undertaken as described previously (Norder et al., 1990, 1993). All sera were screened in duplicate for HBV DNA by PCR using primers hep3/hep39 within the S-gene followed by a second round of PCR using primers hep3/hep4 (Norder et al., 1993). Two HBV DNA positive controls were included in each DNA extraction and subsequent PCR. One of the controls containing 10 pg HBV DNA/ml was amplified with visible products with hep3/hep39 in the first PCR, and the second control containing 0.1 pg HBV DNA/ml was amplified with visible products only after nesting with hep3/hep4. The product obtained with primers hep3/hep39 was used as template in a sequencing reaction with hep3 as sequencing primer (Norder et al., 1993). The pre-core and core gene amplification and sequencing was carried out with primers hepAb (position 1778–1797, with 5′-biotinylation) and hep68 (position 2376–2398). When nesting was necessary, the pre-core/core gene was first amplified with primers hepA/hep66 (position 2487–2509) followed by a second PCR with primers hepAb/hep68. The amplification with both primer pairs was undertaken for 40 cycles with annealing for one minute at 55°C, extension for two minutes at 72°C and denaturation for one minute at 94°C. The PCR product amplified with hepAb/hep68 was purified with Wizard PCR preps DNA purification system (Promega) and used for solid-phase DNA sequencing. The purified product was immobilized on magnetic beads (Dynabeads M280-strepavidin; Dynal AS) and subsequently denaturated to obtain single stranded DNA (Leitner et al., 1995). The sequencing reaction was carried out for both strands using the reagents provided in the Sequenase Version 2.0 DNA Sequencing Kit (USB Cleveland, Ohio), [^{35}S]dATP, 2 units of Sequenase (USB) and the primers used in the PCR as sequencing primers. The

TABLE II. HBV Genotypes of 90 Central American HBV Strains According to Patient Group and HBeAg/anti-HBe Status

		Genotype			
		A	C	D	F
HBeAg positive					
Blood donors	(n = 21)	2 (10%)	0	0	19 (90%)
Patients	(n = 37)				
Acute	(n = 14)	3 (21%)	0	0	11 (79%)
Chronic	(n = 23)	3 (13%)	1 (4%)	4 (17%)	15 (65%)
Pregnant females	(n = 8)	1 (12%)	0	0	7 (88%)
Subtotal	(n = 66)	9 (14%)	1 (1%)	4 (6%)	52 (79%)
Anti-HBe positive					
Blood donors	(n = 11)	3 (27%)	0	0	8 (73%)
Patients	(n = 8)				
Acute	(n = 4)	1 (33%)	0	0	3 (67%)
Chronic	(n = 4)	0	0	0	4 (100%)
Pregnant females	(n = 4)	0	0	1 (25%)	3 (75%)
Subtotal	(n = 23)	4 (18%)	0	1 (4%)	18 (78%)
HBeAg/anti-HBe negative					
Pregnant female	(n = 1)	0	0	0	1
Total	(n = 90)	13 (14%)	1 (1%)	5 (6%)	71 (79%)

sequenced fragments were electrophoresed on 6% polyacrylamide gels and autoradiographed.

RESULTS

Detection of HBV-DNA

After amplification with hep3/hep39 and nesting with the primer pair hep3/hep4, 177 samples were positive with bands of the expected size. The results of the samples are given in Table I.

S gene Sequencing and Genotyping

HBV strains from 90 samples were subjected to limited sequencing to determine the genotype. 66 of the samples contained HBV DNA corresponding to a level of 10 pg/ml or more, 2 of which had anti-HBe. 24 samples were positive for HBV DNA only after nested PCR corresponding to levels between 0.1 to 10 pg/ml, 21 of which had anti-HBe. The genotype for each strain was assessed on the basis of sharing at least 15 of 18 amino acid substitutions between residues 85 to 183 in the HBsAg primary structure known to be conserved for this particular genotype (Table II). The predominant genotype was found to be F (79%), but also genotypes A, D and C, 14%, 6% and 1%, respectively, were present. There were no significant differences in the distribution of genotypes between different groups of patients and countries.

Precore/core Region

The precore and part of the core regions were sequenced in 19 of the 23 strains in sera characterized by the presence of HBV DNA and anti-HBe. Figure 1 gives the nucleotide substitutions at position 1858 and 1896, and details the amino acid substitutions at amino acid residues 1 to 130 in the core region as compared to pHBV-3200 (Valenzuela et al., 1980). Eight strains displayed the wildtype G^{1896}, of which six belonged to genotype F. Six of these samples were positive for IgM anti-HBc. Four of these samples derived from blood donors, one derived from the patient group and one derived from a pregnant female, all of whom had acute hepatitis B. The precore mutation to A^{1896} was found in 11 of the sequenced strains, all of them genotype F strains. Two of these were also IgM anti-HBc positive, one of which derived from a blood donor. In viraemic individuals with anti-HBe, there was a significant correlation between the absence of anti-core IgM in serum and the presence of A^{1896} in viral strains with T^{1858} ($p < 0.01$; Fisher's exact test). A mutation from G to A at position 1899, giving a change from Gly to Asp at residue 29 was found in one genotype F strain. A T^{1858} was found in 16 (all belonging to genotype F) of the 19 strains. This substitution was also found together with the wildtype G^{1896} in five additional F strains from HBeAg positive individuals, one strain from each country.

In the core region, several unique substitutions were identified between amino acid residues 1 to 130. There was a cluster of mutations in the F strains between residues 57 and 68 corresponding to 9.2% of the analyzed region, where 30 out of a total of 95 mutations (32%) were recorded. Six of the samples showed muta-

strain	geno-type	nucleotide position 1858	1896	anti-HBc IgM	1 * 10 * 20 * 30 * 40 * 50 * 60 * 70 * 80 * 90 * 100 * 110 * 120 * 130
pHBV3200	A	C	G	NK	MDIDPYKEFGATVELLSFLPSDFFPSVRDLLDTASALYREALESPEHCSPHHTALRQAILCWGELMTLATWVGNNLEDPASRDLVVNYVNTNVGLKIRQLLWFHISCLTFGRETVLEYLVSFGVWIRTPP
nic2190	A	C	G	-	----------T----------H-----M----------
nic2191	A	C	G	-	----T----------T----------V----------M----------
cr9678H	F	C	G	+	----------S----------D--------T-N----------S----------A----------M----------
*crEP2308	F	T	G	+	----------S----------D--------T-N----------S----------A----------M----------
nic1916	F	T	G	+	----------S----------D--------T-N----------S----------A----------M----------
nic1990	F	T	G	+	----------SE-------P---------I------F-D--------TH----------IF-S---Q------A---------V-M-----K----------
nic2077	F	T	G	+	----------SL-------A--Y----------D--------T-N------T----N-V-NF-S------Q---A----------M----------T-------D--V----------
sal1125	F	T	G	+	----------S----------D----------N----------S----------A----------M----------
cr9090N	F	T	A	-	----------S----------T----D--------T-----I----V--N-V--F-S------D---A----------M----------Q----------
cr9748N	F	T	A	-	----------S--------T-----A----------D------N-T-------HI----------S------Q---A---------HM----------QI----------
cr9909N	F	T	A	-	----------S----------D--------T----------V----VT--GS----------A--------D-HM------F----------
crM359	F	T	A	-	------R----S----------I----D-------GT----------VF-S------D---A---------EHM-----K----------
crSC670	F	T	A	-	----------S----------D----------S----------A---------HM----------I
crSC1634	F	T	A	-	----------S----------D--------T--------V--------N--S----------A--Q--------M---M--Y----------D----------
nic1895	F	T	A	+	----------S----------A----------D--------T--------V----D---RF-S----------A----------M---------Y-----S---DI----------
nic1992	F	T	A	+	----------S----------D--------T--------I-------N--GS----------A----------M---H----------
nic2456	F	T	A	-	------I----S----------D--Q-----T-----I----V----VTAF-S---Q------A----------M----------H----------
hon492te	F	T	A	-	----------SR----------Y----------D--------T-N------YV----------S----------A----------HM----------
hon530te	F	T	A	-	----------S--------V-----I----------D---------S---------V--V-VT---S------Q---A-----D----HM---L---I---V----------F----------
W4B	F	C	G	NK	----------S----------D--------T-N----------S----------A----------M----------
Fou	F	C	G	NK	----------S---F----------I----------D--------T-N----------S----------A----------M----------
9203/85	F	C	G	NK	----------S---F---A----------D--------T-N----------S----------A----------M----------

*This sample had the substitutions characteristics of genotype F in the core region, but derived from a pregnant female doubly infected with HBV strains of genotypes F and D.

Fig. 1. Nucleotide substitutions at positions 1858 and 1896 in the precore region and amino acid substitutions between residue 1 to 130 in the core protein of 19 Central American HBV strains and three previously sequenced genotype F strains, W4B (Naumann et al., 1993), Fou, and 9203/85 (Norder et al., 1994) as compared to pHBV-3200 (Valenzuela et al., 1980). The results from testing for IgM anti-HBc in the 19 sera are also shown. Abbreviations: NK, not known; –, negative; +, positive.

tions in the region corresponding to the CTL epitope between positions 18 and 27. Three of the mutations effected the anchor motif Val at position 27, which was changed to an Ala in two strains and to an Ile in another.

DISCUSSION

The finding that 94% of the HBeAg positive sera investigated from Central America had detectable HBV DNA by PCR is less than reported previously for this category of patients using PCR (Kaneko et al., 1989; Shih et al., 1990). The prevalence of HBV DNA by PCR in the anti-HBe positive samples (27%) is also lower compared to other studies (Shih et al., 1990). Both these discrepancies may be owing to the use of only 0.5 μl of serum in the single PCR according to the procedure (Norder et al., 1990), thus limiting the sensitivity to 2,000 genomes/ml corresponding to an HBV DNA concentration of approximately 0.01 pg/ml. This limitation does not seem to be a disadvantage in this context, since in the original study by Carman et al. (1989) only those carriers with anti-HBe being HBV DNA positive by the dot blot were considered viraemic, despite the fact that 5 of the 7 patients in the anti-HBe positive "nonviraemic" group were positive for HBV DNA by PCR.

All 90 Central American HBV strains could be classified by limited sequencing into the four genotypes, A, C, D and F. The predicted amino acid substitutions for all the strains agreed with the conserved amino acid substitutions of the respective genotype. An unexpectedly high percentage (79%) of the strains were classified as genotype F. Genotype A (14%) was less common. The presence of genotype F in a high proportion of all groups studied suggests that F is the predominant genotype also in native populations of Central America. The predominance of F strains corresponds to the *adw4* predominance of indigenous American populations (Couroucé-Pauty et al., 1983). This subtype has been found to be highly dominating among aboriginal populations of the Amazonia (Gaspar & Yoshida, 1987), and has also been reported to be frequent in some South-American countries such as Argentina (Couroucé-Pauty et al., 1983), and Chile (Castillo et al., 1979). The presence of genotypes A, D and C in Central America is not unexpected considering the immigration from Europe and Asia into the area, and the fact that the subtypes encoded by these genotypes are also present in other regions of the continent (Gaspar & Yoshida, 1987; Laskus et al., 1993).

As mentioned previously it has been shown that the different genotypes of HBV differ with regard to their association with precore mutants depending on the substitutions at position 1858 in the stem of the pregenomic encapsidation signal (Li et al., 1993; Rodríguez-Frías et al., 1995). If there is a T^{1858}, the A^{1896} mutation will enhance the stability of the secondary structure of the encapsidation signal, which will ensure perpetuation of viral replication (Lok et al., 1994). On the contrary, the presence of a C^{1858} generally occurring in genotype A prevents the G to A mutation at 1896, since it will destabilize the stem of the encapsidation signal (Li et al., 1993; Rodríguez-Frías et al., 1995). On the basis of these base pairing requirements it is proposed that genotypes B to D may develop the stop mutation at codon 28 (Li et al., 1993). Thus a high prevalence of precore mutants has been observed in HBV strains belonging to genotype D, which have a T^{1858} (Li et al., 1993; Rodríguez-Frías et al., 1995). So far, the precore sequence has been reported for only three genotype F strains. However, in all of them a C^{1858} is present (Norder et al., 1993; Naumann et al., 1993). Therefore, we anticipated that genotype F strains would not develop the A^{1896} mutation, similar to most genotype A strains. Nevertheless, in this study we found the A^{1896} substitution in 11 anti-HBe positive samples classified as genotype F. Position 1858 in these mutants was a T, which thus explains the presence of A^{1896}, in agreement with previously published data (Li et al., 1993; Rodríguez-Frías et al., 1995). Only one of 17 genotype F strains sequenced had a C^{1858}. To investigate whether the T^{1858} was a mutation or a wild type substitution, i.e. the substitution being present in non-mutant strains, the precore region of five genotype F strains from HBeAg positive samples was also sequenced, which revealed a T^{1858} in all samples. Thus, genotype F strains in Central America have a T^{1858} resembling in this respect strains belonging to genotypes B to E. In previous studies position 1858 has been found to be conserved within genotypes (Li et al., 1993; Rodríguez-Frías et al., 1995). Interestingly with regard to viraemic anti-HBe positive individuals with genotype F strains and a T^{1858}, all five strains lacking the precore mutation derived from patients recovering from acute hepatitis B, while nine of eleven strains with the precore mutation derived from chronic carriers. Thus the majority of anti-HBe positive viraemic carriers with genotype F strains were associated with precore mutants.

There are also other mutations/substitutions in the core region in HBV variants unable to express HBe protein. In our genotype F strains a mutation clustering region was found between residues 57 and 68 of the core protein, which thus differs from previously described hot spots (Chuang et al., 1993; Ehata et al., 1993; Carman et al., 1994; Hur et al., 1996; Lee et al., 1996). According to Ehata et al. (1993) the mutation clustering regions differ between genotypes, and this author discriminates between core genotype 1, representing the core sequence of genotype C and most genotype B strains, and core genotype 2, representing the core sequence of genotypes A and D. The mutation clustering regions were located between residues 84 and 99, and between residues 48 and 60 for core genotypes 1 and 2, respectively (Ehata et al., 1993). Genotype F shares most of the conserved residues in this region with core genotype 2. However, the clustering region in this study only partially overlapped with that of core genotype 2. Therefore, genotype F may have a unique clustering region. None of the mutations in the core

region affected the anchor motif Leu[19], but the Val at position 27 was changed to an Ala in two strains and to an Ile in another one, substitutions which should not affect the anchoring ability, however (Bertolletti et al., 1993). The major conclusions from this study are that Central American HBV strains in general belong to genotype F, that these strains may carry the precore stop mutation and also that the F strains may have a unique clustering region for mutations in the nucleocapsid protein.

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REFERENCES

Bertoletti A, Chisari FV, Penna A, Guilhot S, Galati L, Missale G, Fowler P, Schlicht H-J, Vitielo A, Chesnut RC, Fiaccadori F, Ferrari C (1993): Definition of a minimal optimal cytotoxic T-cell epitope within the hepatitis B virus nucleocapsid protein. Journal of Virology 67(4):2376–2380.

Brunetto MR, Stemler M, Schödel F, Will H, Ottobrelli A, Rizzetto M, Bonino F (1989): Identification of HBV variants which cannot produce precore derived HBeAg and may be responsible for severe hepatitis. Italian Journal of Gastroenterology 21:151–154.

Brunetto MR, Giarin MM, Oliviere F, Chiaberge E, Baldi M, Alfarano A, Serra A, Saracco G, Verme G, Will H, Bonino F (1991): Wild-type and e antigen-minus hepatitis B viruses and course of chronic hepatitis. Proceeding of National Academy of Science USA 88: 4186–4190.

Carman WF, Jacyna MR, Hadziyannis S, Karayannis P, McGarvey MJ, Makris A, Thomas HC (1989): Mutation preventing formation of hepatitis B e antigen in patients with chronic hepatitis B infection. Lancet ii:588–590.

Carman WF, Ferrao M, Lok ASF, Ma OCK, Lai CL, Thomas HC (1992): Precore sequence variation in Chinese isolates of hepatitis B virus. Journal of Infectious Diseases 165:127–133.

Carman W, Thomas H, Domingo E (1993): Viral genetic variation: hepatitis B as a clinical example. Lancet 341:349–353.

Carman WF, Thursz M, Hadziyannis S, McIntyre G, Colman K, Gioustoz A, Fattovich G, Alberti A, Thomas HC (1995): Hepatitis B e antigen negative chronic active hepatitis: hepatitis B virus mutations occur predominantly in known antigenic determinants. Journal of Viral Hepatitis 2:77–84.

Castillo D, Armas-Merino R, González MC, Wolff C, Soto JR, Meléndez M (1979): Subtipos de antígeno de superficie de la hepatitis B (HBsAg) en portadores asintomaticos y pacientes de hepatitis B en Chile. Revista Médica de Chile 107, 373.

Chuang W-L, Omata M, Ehata T, Yokosuka O, Ito Y, Imazeki F, Lu S-N, Chang W-Y, Ohto M (1993): Precore mutations and core clustering mutations in chronic hepatitis B virus infection. Gastroenterology 104:263–271.

Couroucé-Pauty AM, Plancon A, Soulier JP (1983): Distribution of HBsAg subtypes in the world. Vox Sanguinis 44:197–211.

Ehata T, Omata M, Chuang WL, Yokosuka O, Ito Y, Hosoda K, Ohto M (1993): Mutations in core nucleotide sequence of hepatitis B virus correlate with fulminant and severe hepatitis. Journal of Clinical Investigation 91:1206–1213.

Gaspar AMC & Yoshida CFT (1987): Geographic distribution of HBsAg subtypes in Brazil. Memorias do Instituto Oswaldo Cruz 82: 253–258.

Hasegawa K, Huang J, Rogers SA, Blum HE, Liang J (1994): Enhanced replication of hepatitis B virus mutant associated with an epidemic of fulminant hepatitis. Journal of Virology 68(3):1651–1659.

Hoofnagle JH, Dusheiko GM, Seeff LB, Jones EA, Waggoner JC, Bales ZB (1981): Seroconversion from hepatitis B e antigen to antibody in chronic type B hepatitis. Annals of Internal Medicine 94:744–748.

Hunter WM (1978): Radioimmunoassay. In Weir DM (ed.): Handbook of Experimental Immunology. Vol I Immunochemistry, 3rd ed. Oxford: Alden Press, pp 14.1–14.40.

Hur GM, Lee YI, Suh DJ, Lee JH, Lee YI (1996): Gradual accumulation of mutations in precore core region of HBV in patients with chronic active hepatitis: Implications of clustering changes in a small region of the HBV core region. Journal of Medical Virology 48:38–46.

Kaneko S, Miller RH, Feinstone SM, Unoura M, Kobayashi K, Hattori N, Purcell RH (1989): Detection of serum hepatitis B virus DNA in patients with chronic hepatitis using the polymerase chain reaction assay. Proceeding of National Academy of Science USA 86: 312–316.

Kosaka Y, Takase K, Kojima M, Shimizu M, Inoue K, Yoshiba M, Tanaka S, Akahane Y, Okamoto H, Tsuda F, Miyakawa Y, Mayumi M (1991): Fulminant hepatitis B: induction by hepatitis B virus mutants defective in the precore region and incapable of encoding e antigen. Gastroenterology 100:1087–1094.

Laskus T, Persing DH, Nowicki MJ, Mosley JW, Rakela J (1993): Nucleotide sequence analysis of the precore region in patients with fulminant hepatitis in the United States. Gastroenterology 105: 1173–1178.

Laskus T, Rakela J, Persing D (1994): The stem-loop structure of the cis-encapsidation signal is highly conserved in naturally occurring hepatitis B variants. Virology 200:809–812.

Lee YI, Hur GM, Suh DJ, Kim SH (1996): Novel pre-C/C gene mutants of hepatitis B virus in chronic active hepatitis: naturally occurring escape mutants. Journal of General Virology 77:1129–1138.

Leitner T, Escanilla D, Marquina S, Wahlberg J, Broström C, Hansson HB, Uhlén M, Albert J (1995): Biological and molecular characterization of subtypes D, G and A/D recombinant HIV-1 transmission in Sweden. Virology 209, 136–146.

Li JS, Tong SP, Wen YM, Vitvitski L, Zhang Q, Trepo C (1993): Hepatitis B virus genotype A rarely circulates as an HBe-minus mutant: Possible contribution of a single nucleotide in the precore region. Journal of Virology 67:5402–5410.

Liang TJ, Hasegawa K, Rimon N, Wands JR, Ben-Porath E (1991): A hepatitis B virus mutant associated with an epidemic of fulminant hepatitis. The New England Journal of Medicine 324(24):1705–1709.

Lok A, Akarca U, Greene S (1994): Mutations in the pre-core region of hepatitis B virus serve to enhance the stability of the secondary structure of the pre-genome encapsidation signal. Proceeding of the National Academy of Sciences USA 91:4077–4081.

Milich DR, McLachlan A (1986): The nucleocapsid of hepatitis B virus is both a T-cell-independent and a T-cell-dependent antigen. Science 234:1398–1401.

Naumann H, Schaefer S, Yoshida CFT, Gaspar AMC, Repp R, Gerlich W (1993): Identification of a new hepatitis B virus (HBV) genotype from Brazil that expresses HBV surface antigen subtype adw4. Journal of General Virology 74:1627–1632.

Niel C, Moraes MTB, Gaspar AMC, Yoshida CFT, Goméz SA (1994): Genetic diversity of Hepatitis B virus strains isolated in Rio de Janeiro, Brazil. Journal of Medical Virology 44:180–186.

Norder H, Hammas B, Magnius LO (1990): Typing of hepatitis B virus genomes by a simplified polymerase chain reaction. Journal of Medical Virology 31:215–221.

Norder H, Hammas B, Löfdahl S, Couroucé AM, Magnius LO (1992): Comparison of the amino acid sequence of nine different serotypes of hepatitis B surface antigen and genomic classification of the corresponding hepatitis B virus strains. Journal of General Virology 73:1201–1208.

Norder H, Hammas B, Lee SD, Bile K, Couroucé AM, Mushahwar K, Magnius LO (1993): Genetic relatedness of hepatitis B viral strains of diverse geographical origin and natural variations in the primary structure of the surface antigen. Journal of General Virology 74:1341–1348.

Norder H, Couroucé A-M, Magnius LO (1994): Complete genomes, phylogenetic relatedness, and structural proteins of six strains of the hepatitis B virus, four of which represent two new genotypes. Virology 198:489–503.

Okamoto H, Tsuda F, Sakugawa H, Sastrosoewignjo R, Imai M, Miyakawa Y, Mayumi M (1988): Typing hepatitis B virus by homology in nucleotide sequence: Comparison of surface antigen subtypes. Journal of General Virology 69:2575–2583.

Okamoto H, Yotsumoto S, Akahane Y, Yamanaka T, Miyazaki Y, Sugai Y, Tsuda F, Takana T, Miyakawa Y, Mayumi M (1990):

Hepatitis B viruses with precore region defects prevail in persistently infected hosts along with seroconversion to the antibody against e antigen. Journal of Virology 64:1298–1303.

Omata M, Ethata T, Yokosuka O, Hosoda K, Ohto M (1991): Mutations in the precore region of hepatitis B virus DNA in patients with fulminant and severe hepatitis. New England Journal of Medicine 324(24):1699–1704.

Rodríguez-Frías F, Buti M, Jardi R, Cotrina M, Viladomiu L, Esteban R, Guardia J (1995): Hepatitis B virus infection: Precore mutants and its relation to viral genotypes and core mutations. Hepatology 22:1641–1647.

Salminen M, Nykänen A, Brummer-Korvenkontio H, Kantanen ML, Liitsola K, Leiniikki P (1993): Molecular epidemiology of HIV-I based on phylogenetic analysis of in vivo gag p7/p9 direct sequences. Virology 195:185–194.

Shih LN, Sheu JC, Wang JT, Huang GT, Chen JS, Sung JL, Wang TH, Chen DS (1990): Detection of hepatitis B viral DNA by polymerase chain reaction in patients with hepatitis surface antigen. Journal of Medical Virology 30:159–162.

Simmonds P, Smith DB, McOmish F, Yap PL, Kolberg J, Urdea MS, and Holmes EC (1994): Identification of genotypes of hepatitis C virus by sequence comparisons in the core, E1 and NS5 regions. Journal of General Virology 75:1053–1061.

Tong S, Li J, Vitvitski L, Trepo C (1990): Active hepatitis B virus replication in the presence of antiHBe is associated with viral variants containing an inactive pre-C region. Virology 176:596–603.

Valenzuela P, Quiroga M, Zaldivar J, Gray P, Rutter WJ (1980): The nucleotide sequence of the hepatitis B viral genome and the identification of the major viral genes. In: Animal virus genetics, BN Fields, Jaenisch R, Fox CF (eds.). Academic Press, Inc., New York. pp 57–70.